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MECHANISMS OF WOOD DIGESTION IN THE SHIPWORM *BANKIA GOULDI* BARTSCH: ENZYME DEGRADATION OF CELLULOSES, HEMICELLULOSES, AND WOOD CELL WALLS

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Wood in the marine environment is rapidly invaded by bivalve mollusks and isopod crustaceans (Jones and Eltringham, 1971). The bivalves most active in the destruction of sound wood belong to the family Teredinidae (Turner, 1966), commonly called shipworms or teredos. These organisms grow and tunnel rapidly through hard, dry wood while avoiding softer, more rotten regions (Board, 1970). Wood fragments are scraped from the anterior end of the tunnel and enter the digestive tract where they are processed by two structures unique to wood boring mollusks: a very large wood storing caecum and digestive diverticula specialized for wood phagocytosis (Sigerfoos, 1907; Potts, 1923; Morton, 1970).

The unique habitat and digestive tract structure of the Teredinidae suggest two important questions: do shipworms digest wood, and if so, by what mechanisms? Several attempts have been made to demonstrate *in vitro* enzymes active in the digestion of cellulose and wood. Enzymes degrading purified celluloses have been detected (Boynton and Miller, 1927; Greenfield and Lane, 1953; Deschamps, 1953; Nair, 1955a, b, 1956a, b, c; Crosby and Reid, 1971), but in those cases where the cellulose was in a crystalline structure similar to its inferred structure in wood cell walls, the level of activity was extremely low (Kadota, 1959; Boynton and Miller, 1927; Cutter and Rosenberg, 1972; Rosenberg and Cutter, 1973). Production of soluble sugars from ground wood using teredinid extracts has also been reported (Boynton and Miller, 1927; Kadota, 1959).

Wood is not composed solely of cellulose; it is a complex material consisting of cellulose, hemicelluloses, and lignin (Meier, 1964; Northcote, 1972). Cellulose, 40% by weight of the wood cell wall, is a high molecular weight unbranched polymer of β-1,4-D-glucopyranose; the polymers are aggregated into crystalline fibrils (Gardner and Blackwell, 1974a, b; Blackwell and Kolpak, 1975; Stoekmann, 1972). This *native crystalline cellulose* is an anhydrous, water-insoluble material of great stability (Ranby, 1969). The hemicelluloses, about 30% by weight of gymnosperm wood, are a diverse group of non-crystalline hydrophilic polysaccharides partly adsorbed to the cellulose fibrils (Timell, 1964, 1965). The unique properties of wood are conferred by the lignin component (30% of gymonosperm wood), a highly inert and insoluble resin-like compound encrusting the carbohydrates and protecting them from solvent and solute interaction (Pew and Weyna, 1962; Ranby, 1969; Stone, Scallan, Donefer and Ahlgren, 1969; Kirk, 1971).

Some species of fungi and bacteria, together with some insects, gastropod mollusks and ruminant mammals are able to survive and grow on native crystalline

cellulose as a sole carbon source, while other organisms are restricted to non-crystalline cellulose derivatives or forms of cellulose with a more labile crystal structure (Reese, Siu, and Levinson, 1950; Reese and Levinson, 1952; Wood, 1969). In fungi, a complement of diverse hydrolytic enzymes degrades native crystalline cellulose to glucose (Hajny and Reese, 1969). Non-native celluloses are labile to some of these same enzymes. Enzymes active on various β-1,4-glucans, including native cellulose, are termed β-1,4-glucanases (Reese, Maguire and Parrish, 1968; Barras, Moore and Stone, 1969) and may have exo- or endo-activity. In the case of insoluble cellulose particles such as those entering the digestive tract of *Bankia gouldi,* digestive enzymes must produce significant solubilization of the particles if cellulose is to play a nutritionally significant role. The ultimate products of exo- and endo- β-1,4-glucanases will be cellotetraose, cellotriose, and cellobiose, which will be hydrolyzed to glucose by β-glucosidase.

The research reported here was designed to clarify the digestive capabilities of hydrolytic enzymes in extracts of the digestive tract of the teredinid bivalve *Bankia gouldi* Bartsch, assayed as activity producing soluble sugars, principally glucose, from cellulose and wood. The significance of the observed cellulolytic activity for wood digestion was evaluated by comparing *Bankia* tissue extracts to better characterized commercial preparations of cellulolytic enzymes and interpreting the results in the light of our current understanding of wood and cellulose structure. Enzymes with high activity on native crystalline celluloses could not be demonstrated, but β-1,4-glucanase and β-glucosidase activity was readily observed.

Materials and Methods

Specimens of *Bankia gouldi* were collected by immersing pine blocks, purchased from a local lumber yard, under the dock at the Duke University Marine Laboratory, Beaufort, North Carolina. After 1–4 weeks in the sea, the blocks were moved to aerated standing seawater aquaria. The organisms were removed from the wood and the digestive tracts were cut free of the surrounding tissue. The tracts were routinely homogenized in 0.1 M sodium chloride with either a blender or a sonicator, depending on the amount of tissue. A variety of homogenization and extraction media were tested. The homogenates were incubated for up to 12 hours at both 0° C and 37° C in the following solutions: distilled water; 0.1, 0.5, 1.0 M sodium chloride; 0.01 M disodium EDTA; 0.001 M mercaptoethanol; 0.1% Triton X-100; 0.1 M sodium acetate buffers, pH 5.0; 0.1 M sodium borate buffer, pH 9.0. Bacterial growth was inhibited either with sodium azide (0.003 M) or with toluene. In no case was any enhancement of enzyme activity seen over that observed with the 0.1 M sodium chloride.

Homogenates were either dialyzed in acetylated dialysis tubing against 0.02 M sodium phosphate buffer pH 8.0, or centrifuged at 100,000 × *g*, and the supernatant ultrafiltered under pressure on an Amicon PM-10 ultrafilter with the same buffer. A constant problem in this study was obtaining sufficient quantities of tissue extract to characterize the enzyme activity. Shipworms do not actively tunnel throughout the year and they are difficult to remove from wood. As a result, different batches of tissue extract were used at various phases of the study.

To assess the potential nutritional significance of any observed enzyme activity

in the *Bankia gouldi* extract, some parallel experiments were run with cellulose degrading enzymes from other sources. Commercial preparations from better-characterized sources were chosen: "Cellulase" from the fungus *Trichoderma viride* (Worthington Biochemical Co.); "β-glucuronidase" from the gastropod mollusk *Helix pomatia* (Sigma Chemical Co.); and "Cellulase" from the fungus *Aspergillus niger* (Sigma Chemical Co.). These commercial preparations were dissolved in or diluted with distilled water, centrifuged at $100,000 \times g$ and washed on a PM-10 ultrafilter with 0.1 M sodium acetate buffer, pH 5.0.

Substrates for enzyme assays

Cellulose can exist in a variety of forms, and the lability of cellulose polymers to degradation by hydrolytic enzymes is a function of the degree of crystallinity and the precise crystal structure of the substrate (Norkrans, 1967; Rautela and King, 1968; Cowling and Brown, 1969). In addition, as part of the wood cell wall, cellulose is complexed with other potentially protective materials. No single cellulose substrate can provide adequate information on the activity of β-1,4-glucanases. Seven cellulosic substrates were therefore selected to study the influence of crystallinity, and the presence of other molecules on the capabilities of the digestive enzymes.

Milled wood (MW) was prepared from sawdust from the wood used to collect specimens of *Bankia gouldi*. The sawdust was ground in a Wiley mill to pass through a 60 mesh, yielding a fine powder with a particle size distribution somewhat larger than *Bankia* gut contents (based on microscopical observation). *Holocellulose,* wood with the lignin fraction removed, was prepared by the standard sodium chlorite method (Browning, 1967). The resulting white powder was washed thoroughly with water and air-dried. *Alpha-cellulose*, a partially purified wood cellulose, was also prepared by standard procedures, involving extraction with potassium hydroxide (Browning, 1967).

Microcrystalline cellulose (MC) was purchased from Sigma (Sigmacell Type 19). This highly crystalline material is produced by controlled acid hydrolysis of native crystalline cellulose (Halliwell, 1966; Battista, 1971). *Swollen microcystalline cellulose* (SMC), also called Walseth cellulose (Walseth, 1952a), was prepared by suspending MC in 85% cold phosphoric acid (Stone *et al.,* 1969). This treatment increases the hydration of the polymers, separates fibrils, and produces a partial transition to an alternate, folded chain crystal structure (Warwicker, 1971; Stone *et al.*, 1969). *Carboxymethyl cellulose* (CMC) was purchased (Sigma). This is a substituted, non-crystalline and fully water soluble β-1,4-glucan (Eriksson, 1969). *Cellobiose*, the disaccharide unit of cellulose, was also purchased (P–L Laboratories).

Enzyme assay methods

Cellulose and wood degradation were measured as production of soluble products from the substrate. Tissue extracts were assayed for solubilizing activity on the various substrates at 37° C; the choice of temperature was arbitrary. Standard assay conditions were: 0.05 M sodium phosphate buffer, pH 6.4 or 7.0; 0.003 M sodium azide; and substrate concentrations as indicated in Table I. Substrate-free

and protein-free controls were run for all experiments. Incubations were terminated by heating to 100° C, and the residual particulate material removed by centrifugation. A sample of the supernatant was removed for the determination of soluble products. Total reducing sugars were measured with the Somogyi-Nelson technique (Nelson, 1944; Somogyi, 1952) using glucose as a standard; results are expressed as micromoles of glucose-equivalents per milliliter. Glucose was measured enzymatically with glucose oxidase, horseradish peroxidase, and o-dianisidine (Sigma) (Okuda and Miwa, 1973). Protein concentration in tissue extracts was determined by the Lowry technique. One unit (U) of enzyme activity was defined as the production of one micromole of soluble product per minute. *Specific activity* was defined as units per milligram protein (U/mg protein).

The influence of pH on initial rate of hydrolysis assayed as glucose production was studied using 0.05 M sodium citrate and sodium phosphate buffers. Enzyme stability as a function of pH in the absence of substrate was measured by incubating dialyzed *Bankia* supernatant in the same buffers for 24 hours at 4° C and then assaying under the standard conditions at pH 7.0.

Assays over time were run by removing samples from incubations at the indicated intervals and determining the products present. The commercial enzyme preparations were assayed in 0.05 M sodium acetate buffer, pH 5.0, with 0.003 M sodium azide and processed as above.

Results

Homogenates of *Bankia gouldi* digestive tracts produced glucose from all cellulose substrates tested (Table I). The specific activity, standardized for unit weight of substrate, is an index to the relative lability of the substrates to enzyme degradation. SMC is clearly the most labile substrate while MC is the least labile. The lability of MW appears to be even lower (Table II); but since MW is only about 70% carbohydrate (Dean, 1976), the specific activity per milligram carbohydrate is 0.008. All the glucose-producing enzyme activity was soluble and remained in the supernatant after centrifugation.

Preliminary characterization of the glucose producing activity was carried out using dialyzed supernatant material as a source of enzyme. The optimum pH for

Table I

Specific activities of dialyzed Bankia *homogenate on cellulose substrates. Specific activity is in terms of units* (U) *per milligram protein, where one unit is 1.0 micromole of glucose produced per minute from the standard substrate concentration. Standard assay conditions, pH 6.4; 10 minute incubations.*

Substrate	Abbreviation	Standard Concentration (mg/ml)	Specific activity per mg substrate ± standard deviation
Microcrystalline cellulose	MC	5	0.27 ± 0.09
Swollen MC	SMC	1.7	14.90 ± 1.50
Carboxymethyl-cellulose	CMC	5	7.70 ± 0.40
Milled wood	MW	10	—
Cellobiose	—	3.4 (1 micromole/ml)	554.00 ± 44.00

TABLE II

Rates of glucose production from cellulose substrates by different enzyme systems. Rate is expressed as activity units (U); *one unit is one micromole of glucose produced per minute from the standard substrate concentrations. Specific activity is units per mg protein. Initial rate was measured over the first 15 minutes of the reaction; final rate over the last 2 hours. Data is from experiments shown in Figure 1.*

Enzyme source	Substrate	Rate $\times 10^{-3}$ (U/mg substrate)	Specific activity $\times 10^{-3}$ (U/mg protein)	Time period for rate calculation
Trichoderma	SMC	5.53	19.882	Final
	MC	0.12	0.488	Final
	MW	0.02	0.083	Final
Helix	SMC	4.47	16.000	Final
	MC	0.54	0.190	Final
	MW	0.15	0.054	Final
Aspergillus	SMC	27.82	46.410	Initial
	MC	0.01	0.016	Final
	MW	0.003	0.005	Final
Bankia	SMC	12.65	23.235	Initial
	MC	0.014	0.024	Final
	MW	0.005	0.009	Final

glucose production from SMC was found to be between pH 6.0 and 7.0, which was also the region of maximum enzyme stability in the absence of substrate. The maximum rate of glucose production was observed at 40° C, when tested over a range of 20–50° C. The detection of β-1,4-glucanase activity, necessary for SMC degradation, is in part a function of β-glucosidase. The stability of the latter enzyme was found to be sensitive to the buffer used, but had a broad stability over the range of pH 4.0 to 8.0, sufficiently active to hydrolyze any cellobiose produced from SMC hydrolysis. The rate of β-glucosidase activity was maximum at 50° C, suggesting that β-glucosidase is not directly responsible for SMC degradation.

The rate of glucose production from cellulose and cellobiose showed a linear increase when increasing amounts of tissue extract protein were added, as is characteristic for enzymes. The substrate concentration was also related linearly to the rate of glucose production from all substrates. The enzyme activity could not be saturated with substrate using MC or CMC up to levels of 70 mg substrate per mg tissue extract protein. However, SMC saturated the available enzymes at a much lower level, 9.6 mg SMC per mg extract protein; cellobiose saturated β-glucosidase activity at still lower levels. The relationship between the rate of glucose production and the substrate concentration, when standardized for unit weight of protein added, also provides an index to relative substrate lability. The failure of CMC and SMC to saturate available enzymes even at very high substrate levels shows them to be relatively inert to the enzymes involved.

The activity of *Bankia gouldi* tissue homogenate through time on SMC, MC, and MW was compared to the activity of commercial enzyme preparations. With the *Bankia* extract, the substrates were saturated with enzyme at the beginning of the incubation period, and β-glucosidase levels were sufficiently in excess so that all soluble products of substrate hydrolysis would appear as glucose.

Enzymes from *Trichoderma viride* and *Helix pomatia* produced glucose at a

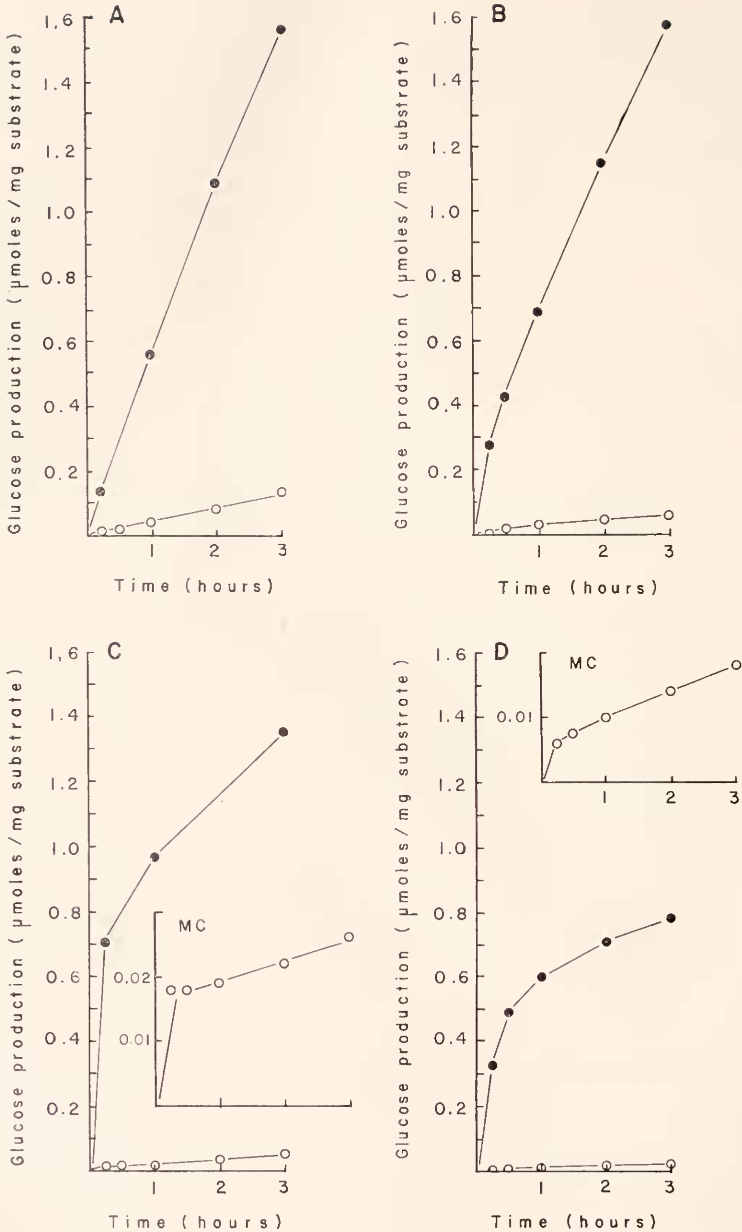

FIGURE 1. Glucose production over a three hour period from cellulose substrates by four enzyme preparations. Standard substrate concentrations at 37° C: A, *Trichoderma viride* enzymes, assayed in 0.05 M sodium acetate buffer, pH 5.0; B, *Helix pomatia* enzymes, assayed in 0.05 M sodium citrate-sodium phosphate buffer, pH 5.0; C, *Aspergillus niger* enzymes, assayed in 0.05 M sodium acetate buffer, pH 5.0; and D, *Bankia gouldi* dialyzed homogenate, assayed in 0.05 M sodium phosphate buffer, pH 6.4. Solid circles represent SMC; open circles, MC.

nearly linear rate through time from SMC, MC, and MW (Figs. 1A, 1B; Table II). This pattern is typical for organisms capable of growth on native crystalline cellulose; the linear rate persists for days *in vitro* (Halliwell, 1966; Dean, unpublished). Both MC and MW were attacked at significantly slower rates, as is consistent with the relative substrate lability. The pattern of activity on these two native substrates is not the same for the two species, however. When the activity is expressed in terms of glucose produced per milligram of carbohydrate, *Helix* enzymes show a MW to MC ratio of 0.46, as compared to a ratio of 0.26 for *Trichoderma viride*. This can be interpreted as showing MW to be relatively more labile to *Helix* enzymes.

The results observed with *Aspergillus* and *Bankia* enzymes show a quite different pattern of activity over time (Figs. 1C, 1D; Table II). Glucose production from SMC is initially rapid and then gradually declines. A similar burst seen with MC and MW is followed by a low, but constant rate of glucose production. Although the activity of these two preparations on SMC was higher than that observed with *Trichoderma* and *Helix*, the rates of glucose production on native crystalline cellulose (MC) were an order of magnitude lower than with the latter two preparations. *Bankia gouldi* extract did show a higher level of activity on MC relative to SMC than did *Aspergillus* enzymes (Table II).

The declining rate of glucose production over a three hour period from SMC by *Bankia* enzymes can be explained as a result of gradual enzyme inactivation (proteases are probably present in the crude extracts), product inhibition, or the disappearance of a more labile fraction of the substrate to yield an inert residue. The results in Figure 2 test for these alternatives. After incubating SMC with *Bankia*

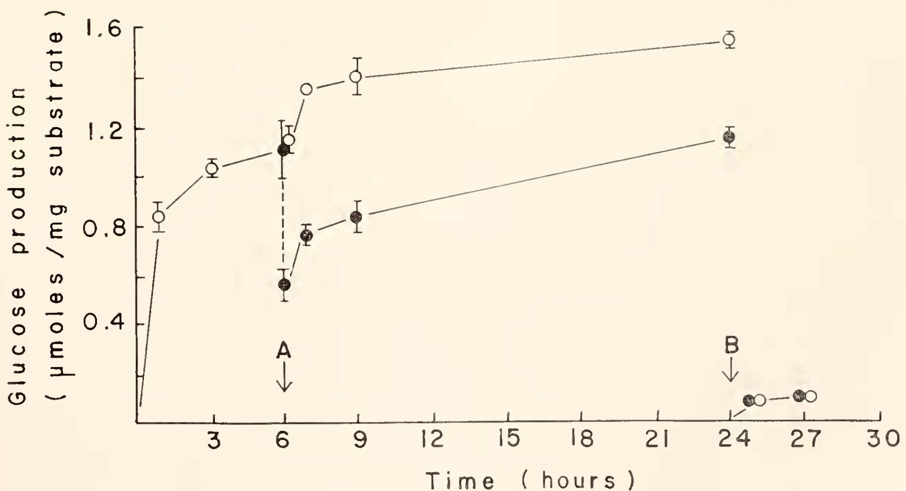

FIGURE 2. Glucose production over time from SMC by *B. gouldi* dialyzed homogenate, standard assay conditions, pH 7.0. Vertical bars indicate standard deviation. At A, the reaction was halted by lowering temperature to 0° C. At B, the reaction was halted by heating the same tubes to 100° C. Residual substrate was washed until glucose-free, and then re-incubated with fresh homogenate. Solid circles represent medium diluted 1:1 with buffer, no substrate removed, and fresh homogenated added; open circles, fresh homogenate added.

extracts for six hours, fresh extract was added, producing a second burst of glucose production whether or not soluble products had been removed. When the substrate was then washed by centrifugation after more extended incubation, and fresh *Bankia* extract added, the subsequent rate of glucose production is comparable to that seen on untreated MC (Fig. 1).

The lability of wood and wood fractions to *Bankia* enzymes was also measured, since this material is the natural substrate for digestive enzymes. The MW used

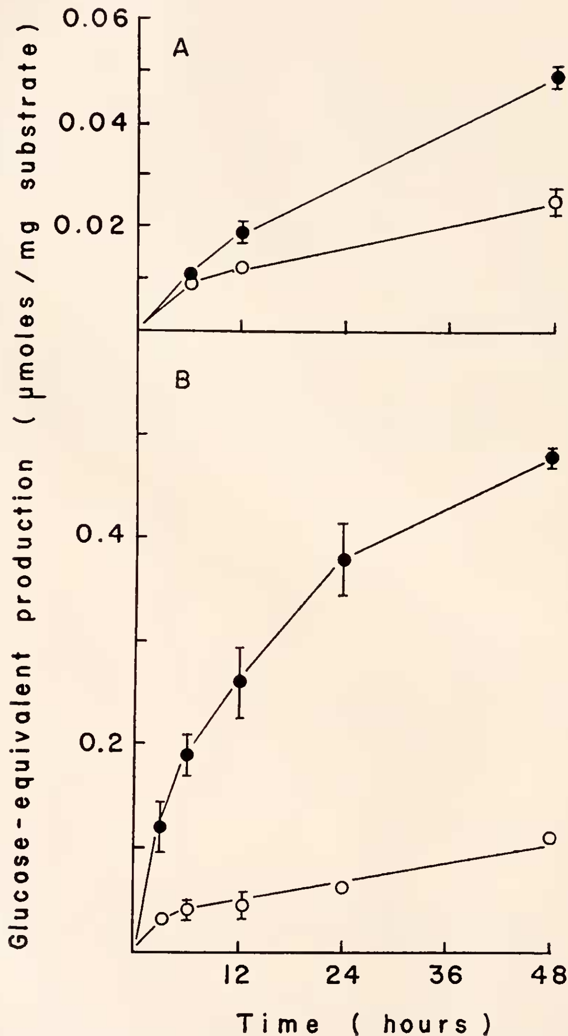

FIGURE 3. Production of soluble sugars over time by *B. gouldi* dialyzed homogenate: standard assay conditions, pH 7.0. A, MW, 37 mg substrate/mg protein; B, delignified MW (holocellulose), 37 mg substrate/mg protein. Solid circles represent total reducing sugars; open circles, glucose.

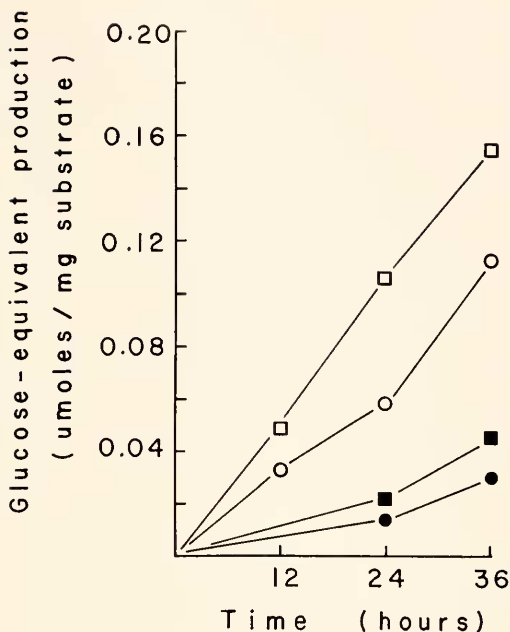

FIGURE 4. Production of soluble sugars by *B. gouldi* dialyzed homogenate from milled wood (squares) and α-Cellulose (circles). The substrate (ordinate) refers to mg carbohydrate in the substrate particles. Both glucose (*closed symbols*) and total reducing sugar (*open symbols*) production are shown.

in these studies is about 70% carbohydrate (Dean, 1976) of which about 60% is native cellulose (Meier, 1964). The observed rate of glucose production from this material by *Bankia* enzymes is consistent with the crystallinity of wood cellulose (Fig. 3A). The higher rate of total reducing sugar production from MW contrasts with results for purified celluloses, where rates of reducing sugar and glucose production are identical, indicating glucose to be the major product.

Removing the lignin fraction from wood greatly enhances the lability of MW to *Bankia* enzymes (Fig. 3B). If the hemicellulose fraction is extracted from the delignified wood, the α-cellulose residue shows greatly decreased lability, here expressed as sugar production per mg carbohydrate in the substrate (Fig. 4). The *Trichoderma* preparation, on the other hand, showed activity on α-cellulose substantially higher than that seen on MW. This α-cellulose residue consists of native crystalline cellulose and glucomannan hemicellulose (Browning, 1967).

DISCUSSION

Available evidence strongly suggests that wood makes an important contribution to the energy requirements of *Bankia gouldi* and other species of wood boring shipworms. The ingestion of wood particles and the uniquely specialized digestive tract argue for an important role. The maintenance of large glycogen reserves (30% of dry tissue weight) in *Lyrodus pedicellatus* is dependent on a supply of wood

(Lane, Posner, and Greenfield, 1952), and shipworms removed from the wood die a few days after emptying the caecum, even if provided with phytoplankton (Turner and Johnson, 1971). This is particularly significant in the light of recent demonstrations that adult specimens of *Bankia gouldi* are capable of suspension feeding (Dean and Back, 1977). Lazier (1924) noted progressive loss of haemotoxylin-positive material from wood fragments during passage through the gut of *Teredo navalis*, which can be interpreted as the loss of the hemicellulose fraction (Freundlich and Robards, 1974). Fecal pellet analyses with *Teredo navalis* (Dore and Miller, 1923) and *Bankia gouldi* (Dean, 1975, 1976) indicate substantial loss of the carbohydrate fraction of wood, including all that is accessible to water soluble enzymes.

The mechanism of wood digestion in shipworms has proved to be as elusive and difficult to study as it is in other organisms, due to present uncertainty about the structure of wood itself. Although the chemistry of most of the cell wall components has been well characterized, the behavior of these components within the wall has not (Timell, 1964, 1965; Northcote, 1972; Stockmann, 1972). The interactions of these fractions are crucial in determining the lability of the material to organisms. In the case of *Bankia gouldi*, it must be asked what relevance any observed enzyme activity has to the requirements of the organism as it tunnels through wood. Therefore, a brief review of current concepts of cell wall structure and degradative mechanisms is necessary.

Wood is a composite material consisting of the cell walls of dead cells. The bulk of wood is composed of fibrous cells characterized by greatly thickened walls which are the main supportive element of trees (Mark, 1967). In softwoods (gymnosperms), as used in this study, the cell is the tracheid; in hardwoods (angiosperms), the fiber cell (Meylan and Butterfield, 1972). These cells may be considered the basic unit of wood.

The wood cell wall has a laminar structure (Mark, 1967; Meylan and Butterfield, 1972). The primary wall is the first to be laid down and is characteristic of all plant cells (Albersheim, 1976). Most of the cell mass in wood, however, is in the secondary wall, characterized by a high density of cellulose fibrils wound helically around the wall and encrusted with matrix material (Mark, 1967). This secondary wall can be looked at as a single cross-linked molecule, resolvable into three phases, corresponding to three chemical fractions: a crystalline phase of cellulose fibrils; a hydrophilic phase of hemicelluloses coating the surface of the crystalline fibrils; and a hydrophobic phase, continuous throughout the wall, in which the other phases are embedded, and consisting of lignin (Dean, 1975, 1976).

The primary emphasis in research on wood digestion in teredinid shipworms and other wood decaying organisms has been on the cellulose fraction. This emphasis is probably a result of the economic importance of wood cellulose as well as the unique problems posed by the structure of the material and the mechanisms of its degradation. It is clear that wood cellulose is in the form of crystalline fibrils (Mark, 1967; Heyn, 1969; Preston, 1971). The best supported model for isolated fibrils appears to be a rigid array of antiparallel polymers linked by hydrogen bonds (Gardner and Blackwell, 1974a, b; Blackwell and Kolpak, 1975) with randomly distributed disruptions and imperfections (Muhlethaler, 1969). Some evidence suggests cellulose in wood can be considered paracrystalline and distinct

in structure from isolated cellulose in ways that would affect its lability to digestive enzymes (Stoekmann, 1972; King, 1968).

In electron micrographs, the crystalline fibrils appear unstained but do have a cortex 10 to 20 Å thick with a high affinity for dyes (Heyn, 1969; Freundlich and Robards, 1974). Since hemicelluloses are known to adsorb strongly to cellulose fibrils (Northcote, 1972), this cortex is assumed to be a layer of hydrophilic hemicelluloses. This very polydisperse group of polymers (Timell, 1964, 1965; Northcote, 1972) has adhesive properties and probably holds the cellulose fibrils together prior to the deposition of lignin (Mark, 1967; Albersheim, 1976).

The crystalline and hydrophilic phases together make up a system of "sticky" fibers wound around the cell; these fibers are embedded in lignin just as the glass fibers in fiberglass are embedded in resin. Lignin is a phenolic polymer forming a three-dimensional, cross-linked gel throughout the wall (Bolker and Brenner, 1970) and is covalently and hydrogen bonded to the hemicelluloses (Northcote, 1972). The hydrophobic nature of lignin (Pew and Wewna, 1962; Kirk, 1971; Wayman and Obiaga, 1974) makes it the critical component in stabilizing the wall because it governs the permeability of the wall to water and the hydration of the hemicellulose fraction (Ranby, 1969; Stone *et al.*, 1969). Lignin also accounts for the high compressive strength of wood (Mark, 1967).

An organism digesting wood cell walls probably will have evolved mechanisms for two critical steps in wood digestion: first, delignification, or exposing the crystalline and hydrophobic phases to enzymes; and secondly, depolymerizing and solubilizing the cell wall constituents themselves. The actual mechanisms by which wood ingesting invertebrates carry out these processes are very poorly understood. Model mechanisms can be developed from the large literature on wood and cellulose–degrading microorganisms, especially fungi (Reese, 1963; Norkrans, 1967; Hajny and Reese, 1969; Kirk, 1973). The major weakness of these studies is that most have dealt with the degradation of isolated cell wall components, usually cellulose, or their derivatives. Frequently the organisms studied are themselves unable to digest whole wood (Merrill, 1966; Wood, 1969). These models do, however, present testable starting points for studying wood-digesting invertebrate organisms. But wood is preeminently a complex macromolecular system, and when this system can be dealt with as a whole, surprises are certain to emerge.

Although some fungi can remove lignin enzymatically (Kirk, 1971), the only mechanism of delignification of demonstrable significance to teredinid shipworms is mechanical disruption of the cell wall. Prolonged milling of wood under various conditions with vibratory or rolling ball mills allows a separation of lignin and carbohydrate (Kirk, 1971; Wayman and Obiaga, 1974). Even short milling times greatly increase the digestibility of the wall by fungal enzymes (Pew and Weyna, 1962; Kirk, 1971). The mechanism probably involves fracturing the lignin matrix away from the carbohydrate phases, exposing them to the medium (Wayman and Obiaga, 1974). One would expect wood digestibility to increase as particle size decreases, as has been shown in soil decay studies (Neal, Bollen, and Lu, 1965).

Hydrophobic and water soluble polysaccharides such as the hemicelluloses are degraded to monosaccharides by the synergistic activity of exo- and endo-hydrolases (Dekker and Richards, 1976). The degradation of cellulose is more problematic. The crystalline fibrils are insoluble in water and display minimal solvent interaction

(Ranby, 1969). Most interior glycosidic linkages are constrained from interacting readily with enzymes, and the hydrogen bonds prevent deformation of the glycosidic linkages, which seems to be an important feature of the catalytic mechanism of at least one endo-hydrolase (Dunn and Bruice, 1973). While the decomposition of cellulose is a biologically unusual process, it is not yet possible to explain how it is unusual at the biochemical level.

As with hemicellulose digestion, the decomposition of native crystalline cellulose at a rate sufficient to support the growth of an organism apparently requires the synergistic activity of several enzymes, each of which may show some solubilizing activity. The classical model for the mechanism of native cellulose degradation supposes that there is a fundamental qualitative difference between the enzyme complement necessary for the degradation of the native substrate and the enzymes required for swollen or soluble cellulose decomposition (Reese *et al.*, 1950). In biological terms, this concept is valid, since some fungi can grow on all celluloses while others are restricted to the more labile forms (Reese and Levinson, 1952; Wood, 1969). This model has proved elusive when pursued biochemically.

Over the past decade, a great deal of effort has gone into the isolation of enzymes from fungal and bacterial sources active on native cellulose. Considerable success has been achieved with the extracellular enzymes released by the fungus *Trichoderma viride* and the wood-rotting fungus *Sporotrichum pulverulentum,* but the results do not fit well with the original model proposed by Reese *et al.* (1950). However, several enzymes have been isolated, which when acting together, extensively degrade native crystalline cellulose to soluble products. Endo-β-1,4-glucanases randomly hydrolyze interior glycosidic bonds, producing chain ends to be attacked by an exo-β-1,4-glucanase producing cellobiose as a principle product (Berghem and Pettersson, 1973; Berghem, Pettersson, and Axio-Fredriksson, 1975, 1976; Eriksson and Pettersson, 1975a, b; Streamer, Eriksson, and Pettersson, 1975). In all cellulolytic microorganisms studied, β-glucosidase catalyzed cellobiose and cellodextrin hydrolysis as the final step in glucose production from cellulose (Berghem and Pettersson, 1974).

The results reported here clearly demonstrate that native crystalline cellulose is relatively resistant to degradation by all enzyme preparations when compared to other forms of cellulose. The patterns of glucose production over time (Fig. 1) are similar for *Trichoderma viride* and *Helix pomatia:* enzymes degrade MC and SMC at a uniform rate through time. *Trichoderma viride* is a soil fungus which can be cultured on native crystalline cellulose as a sole carbon source (Mandels and Reese, 1964), although it cannot grow on wood (Merrill, 1966; Stone *et al.*, 1969). The gastropod mollusk *Helix pomatia* has been shown, like *Trichoderma*, to produce enzymes with high activity on native crystalline cellulose and other plant polysaccharides (Holden, Pirie and Tracey, 1950; Holden and Tracey, 1950; Myers and Northcote, 1959; Koopmans, 1970). That such a similar pattern may be seen in such phylogenetically distant species suggests that the observed pattern may be interpreted as typical for organisms digesting native cellulose at a nutritionally significant rate.

The level of activity of both *Aspergillus niger* and *Bankia gouldi* enzymes on MC was very low but measurable (Fig. 1; Table II), reflecting the very low lability of the substrate. In the case of *Bankia* homogenates, this rate was the

maximum possible with the stated substrate concentration, since the rate of MC solubilization was independent of enzyme concentration; adding additional MC would lead to enhanced glucose production. Since the reaction was substrate limited, the low rate must be a function of a very limited number of sites on the MC particle labile to enzyme attack.

The very high initial rate of glucose production from SMC by both *Bankia gouldi* and *Aspergillus niger* enzyme preparations reflects the enhanced lability of the substrate. The phosphoric acid treatment, which swells the fibrils and alters the crystal lattice (Stone *et al.*, 1969; Warwicker, 1971), probably increases the accessibility of interior glycoside linkages to digestive enzymes. The decline in rate was shown to be in part a function of enzyme inactivation (not including product inhibition), but also a result of decreasing substrate lability (Fig. 2). As the labile fraction is removed, a residual native crystalline core may remain (Walseth, 1952a, b); alternatively, the cellulose chains may rearrange themselves into the more resistant native structure as a result of the degradation (Cowling and Brown, 1969; Stoekmann, 1972). This same pattern has been reported for other preparations (Norkrans, 1950; Walseth, 1952b; Cowling and Brown, 1969).

Aspergillus niger is a fungus called "pseudocellulolytic" by Wood (1969) because of its inability to grow on native crystalline cellulose, while it can grow on swollen and soluble celluloses. The extracellular enzymes, including endo-β-1,4-glucanases (Hurst, Nielson, Sullivan and Shepherd, 1977), have previously been shown to have limited activity on the native substrate but high activity on other β-1,4-glucans (Walseth, 1952a, b; Wood, 1969). The similarity in activity pattern of *Bankia gouldi* enzymes to enzymes from *Aspergillus niger* certainly implies that this level of native crystalline cellulose degradation is insufficient to support the growth and survival of *Bankia gouldi*, and insufficient to explain the extent of cellulose digestion estimated from fecal pellet analysis (Dean, 1975, 1976). The high level of activity on SMC unambiguously demonstrates β-1,4-glucanase activity and implies that both exo- and endo-enzymes are present in *Bankia gouldi* (Berghem and Pettersson, 1973; Berghem *et al.*, 1976). Further conclusions await the isolation of the individual enzymes.

Such enzymes are primarily interesting for their potential role in wood degradation. The lability of wood and wood fractions to *Bankia gouldi* tissue extracts is consistent with the model for the structure of the wood cell wall and with the results obtained with pure cellulose substrates. While the production of glucose and reducing sugars from milled wood (MW) was low, it was greatly enhanced by delignification, emphasizing the protective effect of the hydrophobic phase. Alpha-cellulose, on the other hand, was highly resistant to the *Bankia gouldi* enzymes, as expected for a native crystalline substrate. Since total reducing sugar production was higher than glucose production for all wood substrates tested, enzymes were digesting the hemicelluloses in wood. Even α-cellulose remains contaminated with glucomannan hemicelluloses (Browning, 1967).

If studies on other teredinid species are reinterpreted in the light of concepts presented here, they are consistent with the results on the digestive capabilities of *Bankia gouldi* tissue extracts. β-1,4-glucanase activity ("cellulase") has been reported for *Teredo navalis* (Kadota, 1959; Deschamps, 1953), *Bankia setacea* (Crosby and Reid, 1971), *Bankia indica* (*carinata*) (Nair, 1955a, b, 1956a, b, c),

and *Teredo* (*Lyrodus*) *pedicellatus* (Greenfield and Lane, 1953; Lane and Greenfield, 1952, 1953; Greenfield, 1955). β-glucosidase activity has been described for *Bankia setacea* and appears identical to the activity reported here (Liu and Walden, 1969). *Bankia setacea* (Crosby and Reid, 1971), *Bankia indica* (*carinata*) (Nair, 1955a, b, 1956a, b, c), and *Teredo navalis* (Boynton and Miller, 1927; Kadota, 1959) have been reported to produce reducing sugars from ground wood; enhancement of production after delignification was seen with *Teredo navalis* enzymes (Kadota, 1959).

The β-1,4-glucanase activity reported for *Teredo* (*Lyrodus*) *pedicellatus* (Greenfield and Lane, 1953) showed essentially the same pH behavior seen here with *Bankia gouldi*. A partial purification of a *Lyrodus* enzyme was achieved with an affinity method involving CMC, but the activity was not further characterized (Greenfield, 1955). The *Teredo navalis* enzyme activity also showed similarities to my results with *Bankia gouldi*. However, a bimodal pH-activity curve with "cellulose" as the substrate with optima at pH 5.6 and 6.6 has been reported for *Teredo navalis* (Kadota, 1959). A similar bimodal result has more recently been reported for this species but with optima at 4.6 and 6.1 (Cutter and Rosenberg, 1972; Rosenberg and Cutter, 1973).

In all species of teredinid shipworms investigated, the activity of tissue extracts on native crystalline cellulose was low (Kadota, 1959; Nair, 1955a, b, 1956a, b, c; Boynton and Miller, 1927; Cutter and Rosenberg, 1972; Rosenberg and Cutter, 1973). The only quantitative data is for *Teredo navalis,* where a maximum rate of glucose production from MC of 2×10^{-6} U/mg MC (as calculated from their figures) was recorded (Rosenberg and Cutter, 1973). This activity is more than an order of magnitude lower than the low activity reported here for *Bankia* (Tables I, II). Significant solubilization of native crystalline cellulose *in vitro* has therefore not been demonstrated with any teredinid shipworm, despite claims to the contrary.

In conclusion, the results for *in vitro* enzyme activity from *Bankia gouldi* will be discussed in terms of the mechanisms of feeding and digestion in this and other shipworm species. *Bankia gouldi,* in tunneling through wood, fractures the cell wall into fine particles, ranging down to 10 μ or less (Plate 18, Fig. 1 in Dore and Miller, 1923). This fracturing probably disrupts the lignin matrix and exposes a significant fraction of cell wall polysaccharide, including cellulose. Once ingested, these particles may be stored in the caecum, where they may be exposed to extracellular digestive enzymes. Some portion of these particles may also enter the specialized digestive diverticula and be phagocytosed (Potts, 1923; Morton, 1970), with possible exposure to lysosomal digestive enzymes (Owen, 1974). Hydrolysis of cellobiose and other oligosaccharides by β-glucosidase and other glycosidases may be extra or intracellular.

Since the activity of the hemicellulolytic enzymes producing reducing sugars from wood can be detected at pH 6.0 to 7.0, which is in the normal range for bivalve digestive tracts (Owen, 1974), they may be extracellular enzymes active in the stomach and caecum. The extent to which these enzymes can produce soluble products from wood particles will be limited by the lignin fraction and will be a function of the particle size (Pew and Weyna, 1962; Stone *et al.,* 1969; Neal *et al.,* 1965; Wayman and Obiaga, 1974).

The β-1,4-glucanase activity reported here for *Bankia* is probably active in breaking β-1,4-glycosidic linkages in hemicelluloses, but its role in the digestion of wood cellulose remains uncertain. The failure to demonstrate enzyme activity producing soluble products from native crystalline cellulose at a nutritionally significant rate remains a problem. There are several possible explanations. Fracturing the cell walls and digesting the hemicelluloses should expose a portion of the cellulose to enzyme attack. While these fibrils are crystalline, they may be less crystalline, and therefore more labile, than commercial MC. Rautela and King (1968) emphasize the importance of precise crystal structure to enzyme lability, while King (1968) found that isolated celluloses were less labile to fungal enzymes than intact cell wall cellulose. The possibly paracrystalline nature of the fibrils when within the wall has been emphasized by others (Muhlethaler, 1969; Stoekmann, 1972). The β-1,4-glucanases may in fact solubilize ingested wood particle cellulose to an extent necessary to explain the cellulose content of fecal pellets (Dore and Miller, 1923; Dean, 1976).

A second explanation is that the failure to demonstrate the expected enzyme activity is an experimental artifact. Enzymes may be lost during the extraction procedure. Even in whole homogenates, the enzymes may remain bound to wood fragments in the caecum; some β-1,4-glucanases from *Trichoderma* show strong binding to crystalline celluloses (Mandels, Kostick and Parizek, 1971). Alternatively, the enzymes may simply be inactive under the chosen extraction and assay procedures. If so, they are very different from their fungal counterparts.

In the event that lysosomal enzymes are responsible for cellulose digestion, it is difficult to predict a mechanism. Hydrolases, perhaps active at acid pH, may be involved; an exciting possibility is that non-hydrolytic mechanisms play an important role, and the principle products of digestion are not glucose or reducing sugars. The coupling of cellobiose oxidation to the reduction of lignin quinones is a possibility (Westermark and Eriksson, 1974a, b, 1975), and any cellulose degradation would not be detected by measuring glucose production. Alternatively, other non-hydrolytic reactions, inactive under the assay conditions, may be necessary for maximum hydrolysis (Eriksson, Pettersson and Westermark, 1974). Cellulose digestion may therefore involve as yet undescribed oxidative reactions that are characteristic of intact, metabolically active cells. Elucidating the mechanism may therefore involve formidable technical problems in cell fractionation of the phagocytic cells.

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Summary

Tissue extracts from the digestive tract of the wood-boring bivalve *Bankia gouldi* were tested for their ability to degrade celluloses and wood to soluble sugars.

Tissue extracts showed high levels of glucose production from cellobiose, demonstrating the presence of β-glucosidase.

The crystal structure of cellulose determines its lability to enzymes. Microcrystalline cellulose (MC) was used as an analogue to native crystalline cellulose in wood cell walls. Swollen microcrystalline cellulose (SMC) and carboxymethyl cellulose (CMC) are more labile celluloses. *Bankia goudi* extracts showed maximum activity on SMC and minimum activity on MC, when assayed as glucose production.

When assayed over time, glucose production from SMC gradually declined as a function of enzyme inactivation and decreasing substrate lability. These results are interpreted as showing the removal of a cellulose fraction of altered crystal structure, leaving the more inert native crystalline fraction.

Activity of *Bankia gouldi* extracts over time was compared to commercial cellulose-digesting enzyme preparations. The pattern of activity closely resembles that of *Aspergillus niger,* a fungus unable to grow on native crystalline cellulose. The pattern is very different from that seen with *Trichoderma viride* and *Helix pomatia* enzymes, characterized as true cellulose decomposing systems.

The production of glucose from ground wood by *Bankia gouldi* extracts is consistent with the crystallinity of the substrate and the results for purified cellulose. A higher level of production of reducing sugars other than glucose indicates the digestion of wood hemicelluloses.

Digestibility of wood hemicelluloses is limited by the lignin fraction in the wood cell wall.

The experimental results were interpreted in terms of a model for the molecular architecture of the wood cell wall. The cellulose is present as crystalline fibrils coated with hydrophilic hemicellulose polymers. These coated fibrils are embedded in a hydrophobic lignin matrix. The digestibility of wood is a function of polymer accessibility and lability to digestive enzymes.

The enzymes demonstrated *in vitro* in *Bankia gouldi* tissue extracts are β-1,4-glucanases with limited activity on native crystalline cellulose, which they cannot digest at a nutritionally significant rate. The mechanism of cellulose digestion by shipworms remains an unsolved problem.

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